Macromolecular Structures: Proteins

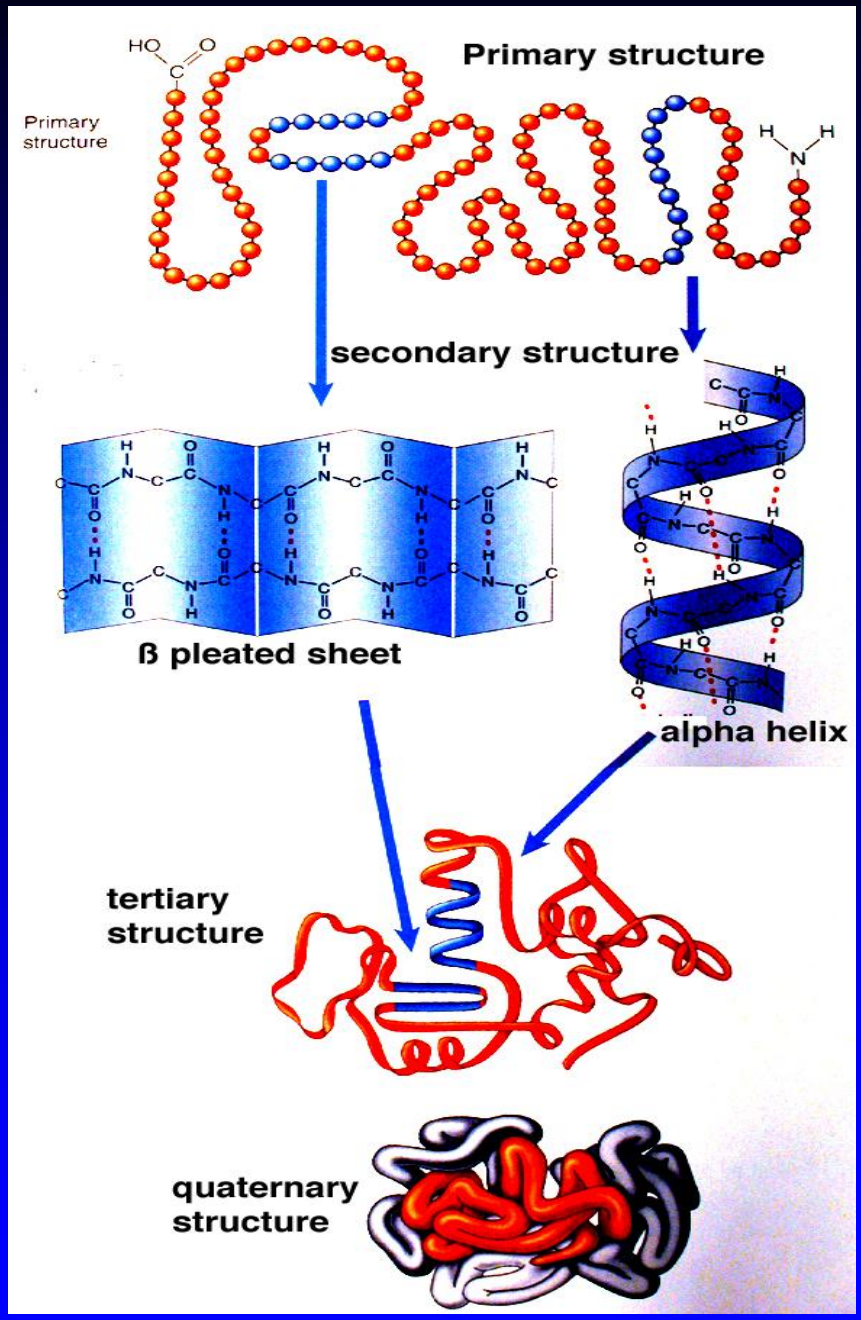
Primary Structure - the linear sequence of amino acids in the polypeptide chain.

Secondary Structure - interactions between the individual amino acids create larger order structures to form discreet regions (α-helices and β-sheets).

Tertiary Structure - the packing of the secondary structures to give a protein's overall structure.

Quaternary Structure - the interaction between several different proteins (or subunits) to give the final, functional protein.

(NOTE - not all proteins contain quaternary structures.)


Primary structure
Primary structure
secondary structure
ß pleated sheet
alpha helix
tertiary structure
quaternary structure

Molecular Methods

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The Development of Molecular Methods

- Understanding the mechanisms of how biological molecules work to drive various biological processes requires the use of molecular techniques.
- Molecular methods are the techniques that are used to study and analyze molecular biological compounds, such as DNA, RNA, and proteins.
- The techniques discussed in this lecture were all developed from the basic understanding of the properties of the biomolecules themselves.
- An understanding of the base pairing characteristics of DNA and RNA allowed the development of hybridization techniques, sequencing, and the Polymerase Chain Reaction (PCR).
- An understanding of the biochemical nature of DNA, RNA, and proteins allowed the development of separation techniques.

DNA Handling

Denaturing and Renaturing the Double Helix

- **Denaturation** - the separating of the two strands of the double helix by heating the solution above physiological temperature or by increasing the pH of the solution.
- **Renaturation** - reversing the de-naturation process by slowly cooling the DNA solution back to physiological temperatures thereby allowing the base pairs to form. The process of re-naturation can allow artificial DNA strands to interact with their complementary target to form a hybrid piece of DNA. This process is called **hybridization** and is the basis for several important techniques.

DNA Handling

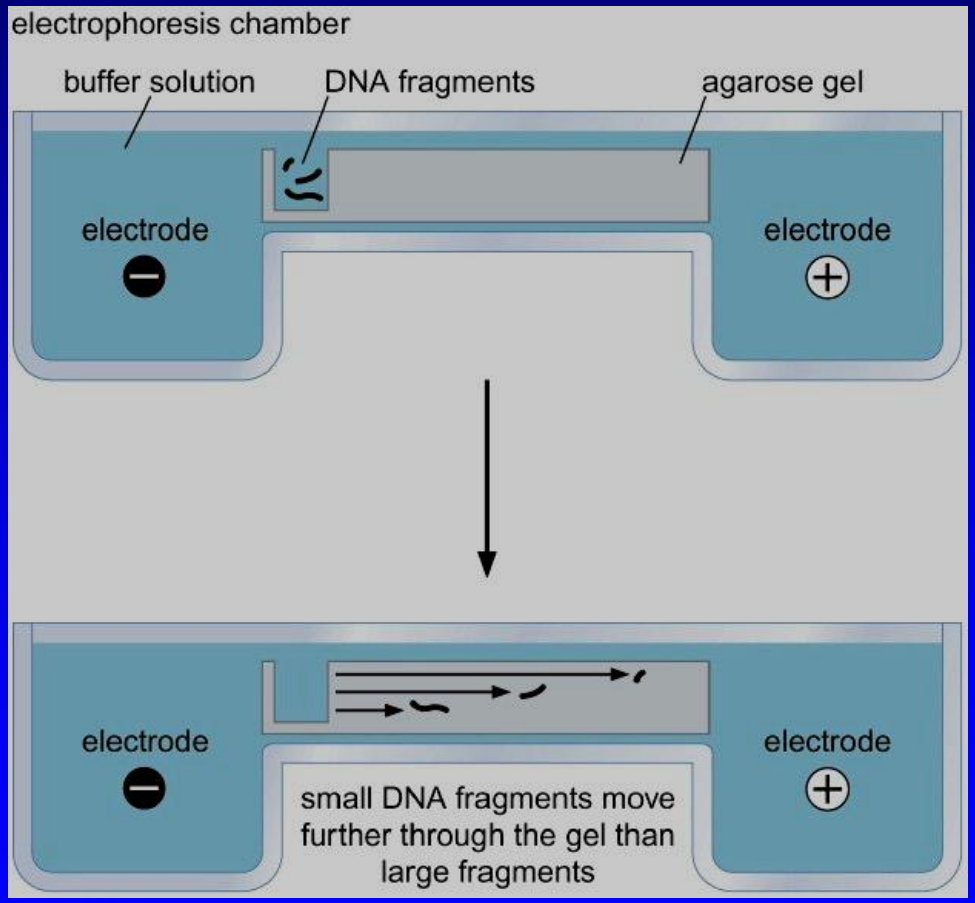
Denaturation is Dependent on Melting Temperature

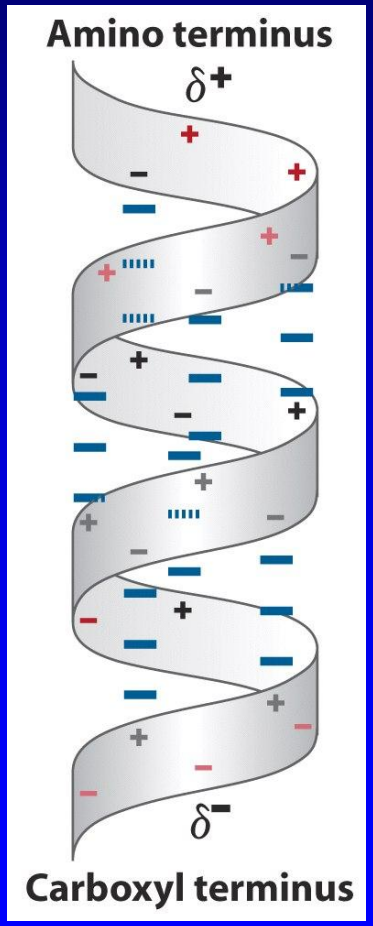
- Duplex DNA absorbs less ultraviolet light than do individual DNA chains. This is a phenomenon known as **hyperchromicity**.
- Measure the absorbance at 260nm (A_{260}) as a function of increasing temperature and watch for an increase in the A_{260}, which will indicate the conversion from double stranded to single stranded DNA.
- **Melting Temperature (T_M)** - the point at which 50% of the DNA has converted from double stranded to single stranded. The melting temperature of a piece of DNA is dependent on DNA sequence and is used to determine annealing temperatures in the polymerase chain reaction (PCR).
- G:C base pairs have three hydrogen bonds, A:T base pairs have two. Therefore, G:C base pairs contribute more to the stability of the DNA.

Simple approximation of T_M:

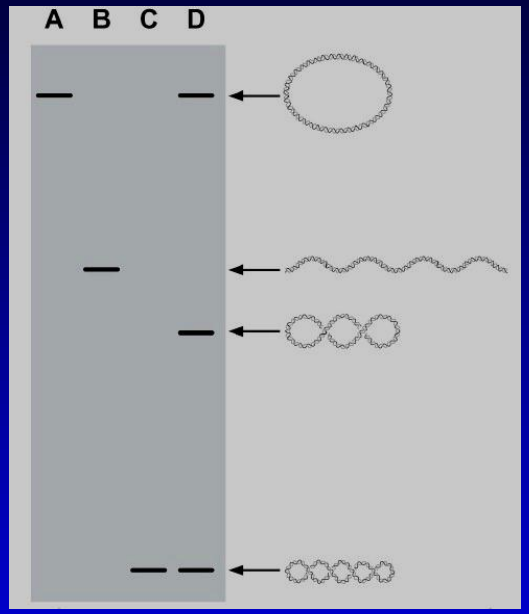
$$T_M = 2(A + T) + 4(G + C)$$

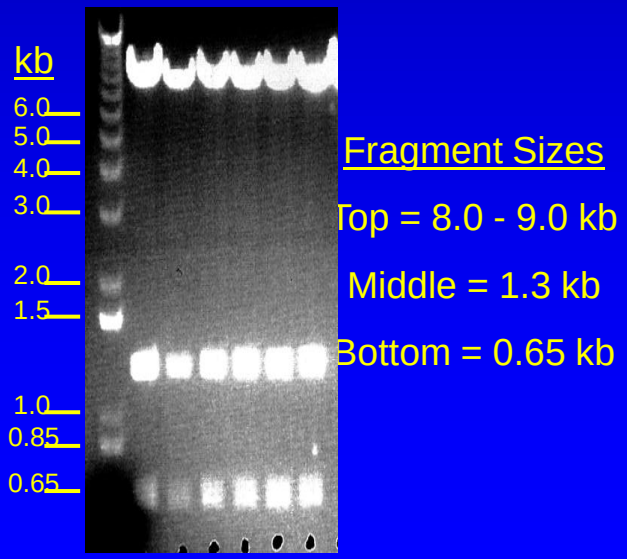
DNA and Gel Electrophoresis





DNA and Gel Electrophoresis





Fragment Sizes

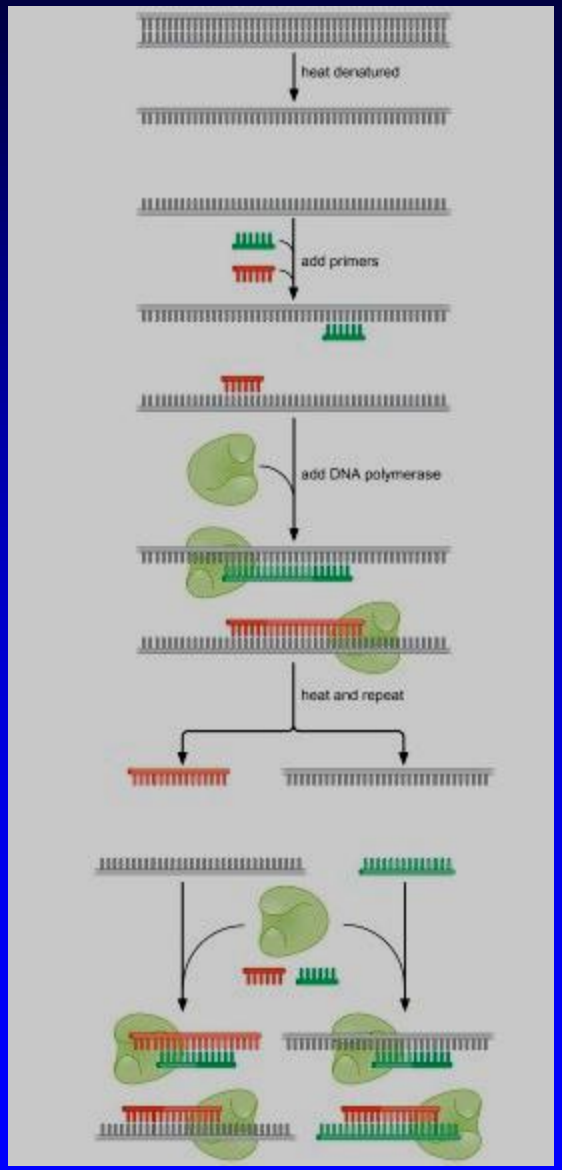
Top = 8.0 - 9.0 kb

Middle = 1.3 kb

Bottom = 0.65 kb

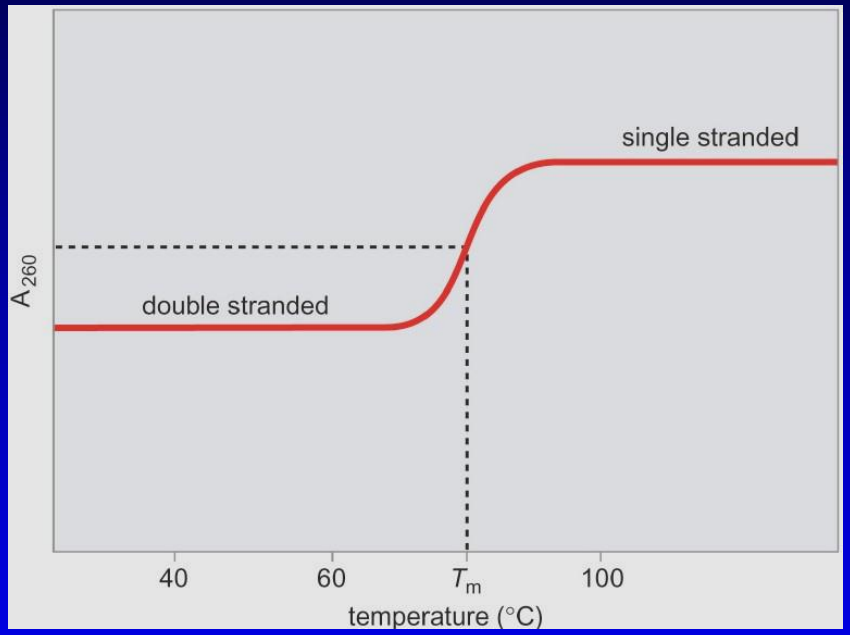
- Agarose (less resolving power, larger size range [several thousand base pairs]) or polyacrylamide (greater resolving power, smaller size range [a few hundred base pairs]) is used as the separating matrix.
- Both matrices create a “sieve-like” solid for the DNA to migrate through.
- DNA is negatively charged (phosphate backbone). When it is subjected to an electric field it will migrate toward the positive electrode.
- Pores in the agarose gel create a sieve-like environment that “regulates” how quickly a piece of DNA will migrate through the gel in the presence of an electrical field.
- The migration of the DNA is dependent on their “effective volume” (for cccDNA) and the number of base pairs (for linear DNA). The larger the linear piece of DNA, the slower it will migrate through the agarose gel.
- Once the gel has been run (i.e. - sufficient time in the electric field to achieve proper separation), the gel is stained with ethidium bromide (EtBr). EtBr is a fluorescent dye that intercalates between the DNA bases and “lights-up” the DNA under a UV light.

The Polymerase Chain Reaction (PCR)



- PCR uses a heat-stable DNA polymerase to synthesize DNA using a specific chemically synthesized oligonucleotide, or **primer**.
- DNA polymerase synthesizes DNA in the 5'- to 3'-direction only.
- Therefore, careful selection of the proper oligonucleotides will allow the specific amplification of the desired fragment of DNA.
- Two oligonucleotides are required - one complementary to the 5'-end of the desired DNA fragment such that synthesis will proceed in the proper direction and one complementary to the 3'-end of the desired DNA fragment.
- The solution containing the **template DNA** (the DNA that is being used as the basis for the amplification), the oligonucleotides, and the DNA polymerase are heated to a high temperature to denature the DNA.
- The solution is quickly cooled to the **annealing temperature** to allow the oligonucleotides to anneal to their target sites.
- The temperature is raised to the optimal for synthesis (often called **extension**).
- The entire procedure is repeated 15 - 40 times resulting in a greatly amplified, highly specific DNA fragment.

The Polymerase Chain Reaction (PCR)



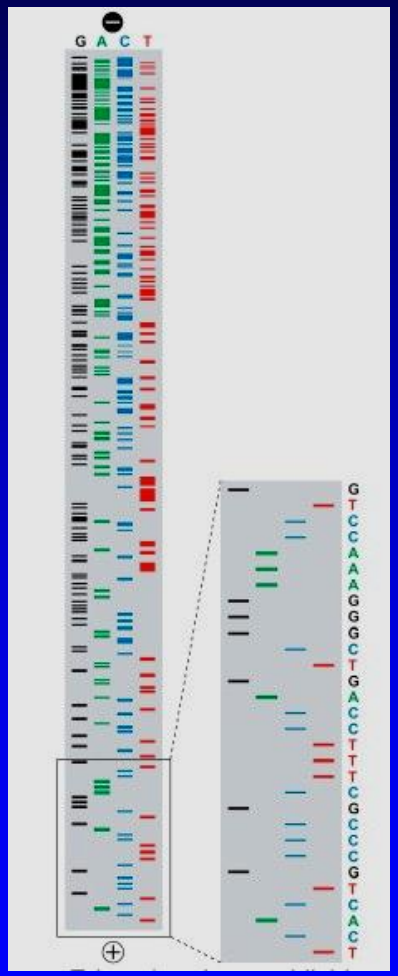
The diagram by which melting and annealing temperatures could be determined.

- **annealing temperature** - the temperature at which the oligonucleotide will specifically anneal with the target sequence on the template DNA. This temperature is based on the T_M of the oligonucleotide and is usually placed about 2°C below the T_M.

- The amplification occurs in a **geometrical fashion**. Each round of amplification will double the amount of DNA from the previous round. If you start with one molecule, you will have two molecules after the first round, four molecules after the second, eight after the third, etc.

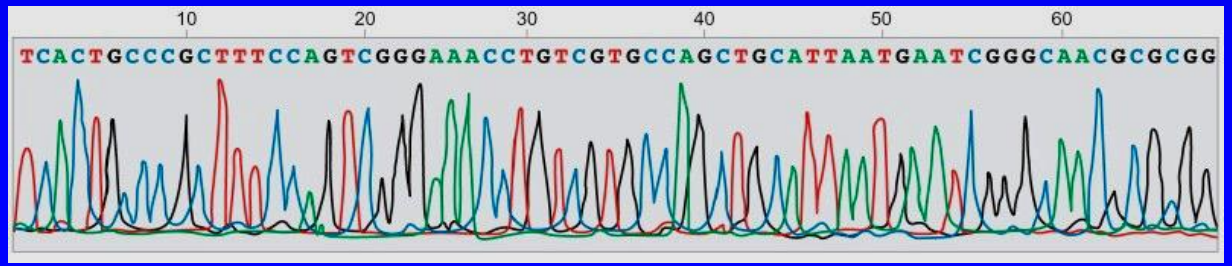
DNA Sequencing

Sequencing of DNA is the way to determine the exact base composition of a gene



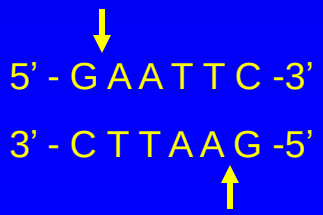
The “old-fashioned” way - one of the nucleotides is radiolabeled. There are four separate reactions, one for each 2’,3’-dideoxy nucleotide. The reactions are then separated by polyacrylamide gel, dried, and exposed to film. The sequence is then read from bottom to top (because the earliest terminators will occur closest to the primer, thereby giving the shortest DNA fragments). This can give, at best, about 200 - 250 bp of good sequence.

The “newer” way - one of the nucleotides is fluorescently labeled, each nucleotide with a different color. There are four separate reactions, one for each 2’,3’-dideoxy nucleotide. The reactions are then separated by column chromatography. The automated sequencing machine reads the color of the nucleotide as it exits the column and identifies that nucleotide. The earlier the nucleotide comes off the column, the smaller the fragment and the closer it is to the primer. This is easier on the researcher, faster, and gives about 650 - 900 bp of good sequence.

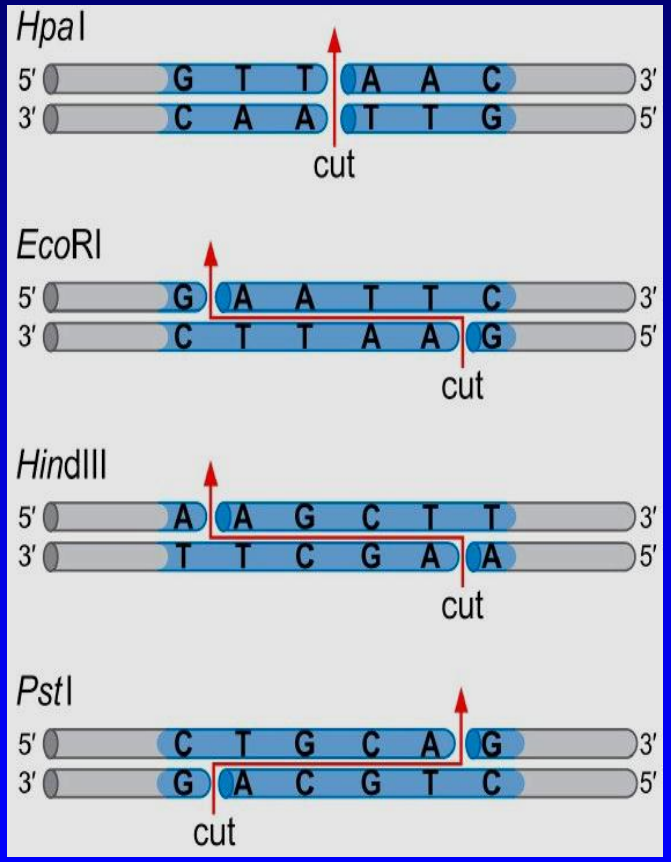


Restriction Enzymes

- **Restriction Exonucleases:** are enzymes that work by cleaving nucleotides one at a time from the end (exo) of a polynucleotide chain.
- **Restriction Endonucleases:** enzymes that recognize a very specific DNA sequence and cleave the DNA at these specific sites.
- Originally isolated from bacteria where bacteria use them to prevent infection by bacteriaphage (or bacterial viruses). They are named based on the organism from which they were isolated (EcoRI - E. coli; BamHI - Bacillus amyloliquefaciens; HindIII - Haemophilus influenzae
- The resulting cleavage pattern of the DNA is known as a **restriction map**.
- The sequences recognized by the enzyme are most frequently palindromic (i.e. - they read the same forward [on the sense strand] and backward [on the antisense strand]). For example, EcoRI recognizes the following site and cleaves at the arrows:



Restriction Endonucleases



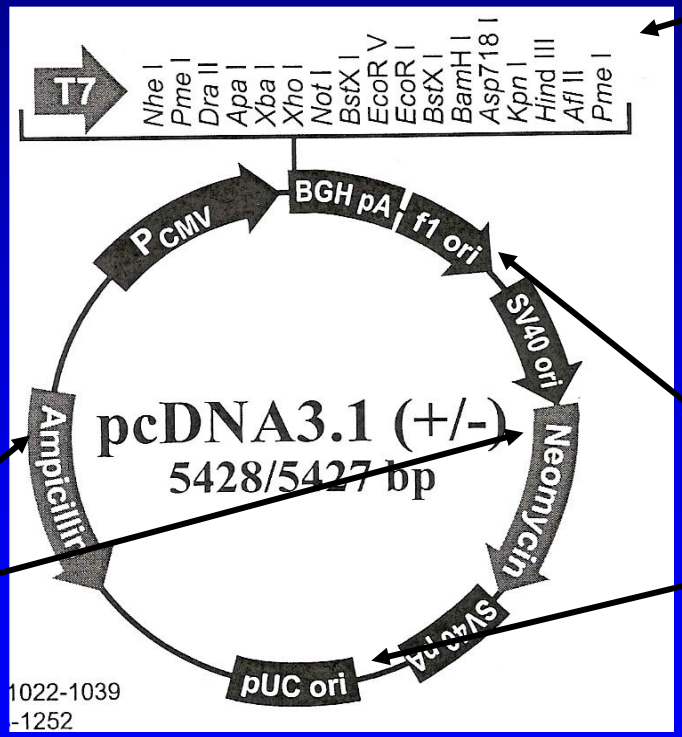
Blunt end

Sticky ends

Cloning DNA into Plasmid Vectors

When working with a defined piece of DNA it is oftentimes necessary to clone the gene (or insert the gene) into a second DNA molecule that allows the propagation of the DNA in bacteria along with a simple transfer to and from bacteria and mammalian cells. These closed circular DNA molecules are called **plasmid vectors**. Plasmid vectors are autonomously replicating pieces of DNA and must have the following characteristics:

Multiple Cloning Site: This is a region that contains restriction sites that are unique within the vector. This allows DNA fragments to be inserted at a defined point within the vector.



Selectable Markers:

These allow for the selection of either bacteria (Ampicillin) or eukaryotes (Neomycin) that have received and contain the plasmid vector.

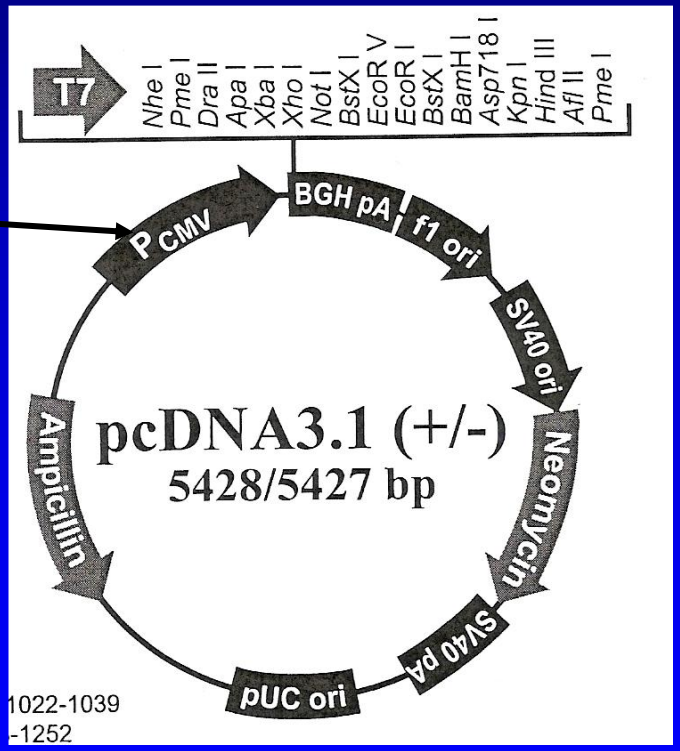
Origins of replication: These allow the vectors to replicate independent of the host. (pUC ori - bacteria; SV40 ori - eukaryotes)

Cloning DNA into Plasmid Vectors

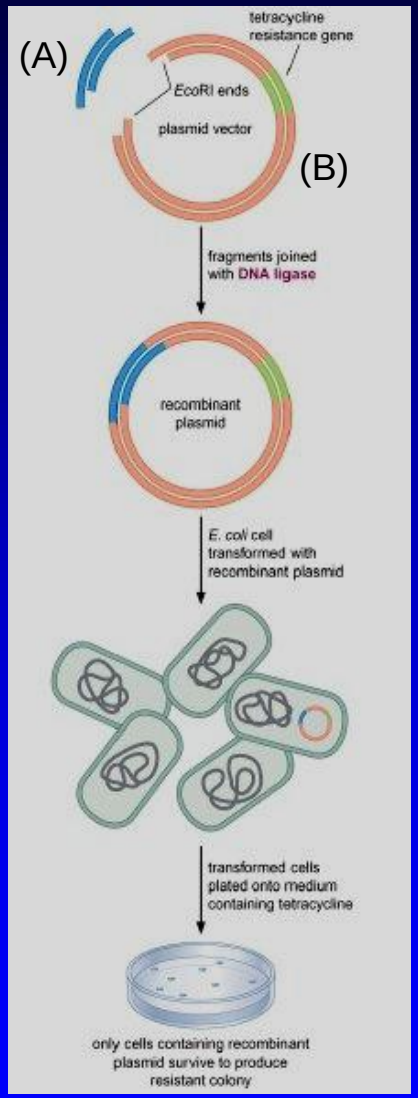
Some plasmid vectors can also be **expression vectors**. These vectors (like the one listed below) contain an additional DNA element called a promoter. These promoter elements can be either from eukaryotic origin (allowing the expression of the desired protein in eukaryotes) or prokaryotic in origin (allowing the expression of the desired protein in prokaryotes).

Eukaryotic Promoter:

The cytomegalovirus (CMV) promoter allows the constitutive (or constantly being expressed) expression of the gene that has been cloned into the MCS immediately downstream of it.



Cloning DNA into Plasmid Vectors



- To clone a piece of DNA into a plasmid vector:
  - Vector (A), which contains the DNA fragment of interest, is digested with restriction enzymes that will release the entire fragment from the vector.
  - The released DNA fragment is separated from the parent vector by agarose gel electrophoresis with subsequent purification of the fragment from the gel.
  - The target vector (B), is also digested with the identical restriction enzymes, which generates a linearized backbone vector with complementary DNA overhangs, and gel purified.
  - The DNA fragment (A) is then joined with the target vector (B) using another enzyme called **DNA Ligase**. [This enzyme creates a covalent bond between the insert DNA and the backbone vector DNA at the overhangs where the two pieces of DNA are complementary].
  - The ligated DNA is transformed into bacteria (that is, chemical or electrical means are used to transfer the ligated DNA into bacteria) and grown on plates that contain ampicillin.
  - Because of the ampicillin resistance gene on the vector, only those bacteria that contain the ligated vector will be able to grow in the presence of the antibiotic.
  - Pick some of the bacterial colonies, isolate the DNA, and confirm the presence of the correctly formed clone by additional restriction digests.

  NOTE: in some cases, only one restriction enzyme is used generating the identical ends on the 5'- and 3'-ends of the insert fragment (**bidirectional cloning**). In these cases it is necessary to use another restriction enzyme to confirm the correct orientation of your cloning.

END